Acta Medica Okayama

Volume 37, *Issue* 2 1983 *Article* 2

APRIL 1983

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KEYWORDS: gamma-aminobutyric acid (GABA) receptor, radioreceptor assay (RRA), rat central nervous system, endogenous inhibitor, Triton X-100

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*PMID: 6307002 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 37 (2), 93–106 (1983)

CHARACTERISTICS OF GAMMA-AMINOBUTYRIC ACID (GABA) RECEPTORS IN THE RAT CENTRAL NERVOUS SYSTEM

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Received November 18, 1982

Abstract. Characteristics of gamma-aminobutyric acid (GABA) were investigated in the rat central nervous system by radioreceptor assay (RRA). Scatchard analysis revealed that the rat brain had two distinct GABA binding sites with an apparent dissociation constant (Kd) of 11.7 nM and 34.7 nM. The highest level of specific [^{3}H]-GABA binding was found in the rat cerebellum. Imidazole acetic acid, a potent GABA agonist, was effective in displacing [^{3}H]-GABA binding but β-alanine was slightly effective in inhibiting [^{3}H]-GABA binding. Muscimol, the most potent GABA agonist, has been used as a ligand to characterize the postsynaptic GABA receptors. However, the maximal binding capacity (Bmax) of muscimol-RRA was about 3 times larger than that of GABA-RRA, suggesting that muscimol might label not only GABA receptors but other unknown receptors as well. An endogenous inhibitor of GABA receptor binding was purified from the P_2 fraction of rat brain with 0.05 % Triton X-100. The endogenous inhibitor was competitive with GABA on GABA binding sites. The inhibition by the endogenous inhibitor of GABA receptor binding was blocked by the allosteric effect of diazepam. In the presence of diazepam, [^{3}H]-GABA binding with the endogenous inhibitor was larger than that with GABA, whereas there was no difference in the absence of diazepam. This indicated that the endogenous inhibitor was not GABA itself. The molecular weight of the endogenous inhibitor was estimated by gel filtration to be less than 3,000 daltons.

Key words : gamma-aminobutyric acid (GABA) receptor, radioreceptor assay (RRA), rat central nervous system, endogenous inhibitor, Triton X-100.

Gamma-aminobutyric acid (GABA) has been established as a major inhibitory neurotransmitter in the invertebrate peripheral and central nervous system (CNS) and in the vertebrate CNS (1-3). Recently, Zukin *et al.* (4) demonstrated a sodium-independent specific binding of GABA to the synaptic membrane fraction of rat brain which appeared to be associated with the postsynaptic GABA receptor. Since then, many studies about GABA receptor binding have been reported (5-8) in which the sodium-independent GABA binding site was identified as a postsynaptic receptor and the sodium-dependent GABA binding site as a neuronal uptake receptor site. Muscimol, the most potent GABA agonist, has become available as a radioligand and has been used to characterize the postsynaptic GABA recep-

tors (9, 10). More recently, several reports (11-13) indicated a presence of an endogenous substance which inhibited [^{3}H]-GABA binding in synaptic membrane fractions of rat brain. Despite many reports about the endogenous inhibitor, its definitive properties are as yet unknown. In the present study, optimum conditions for the GABA radioreceptor assay (GABA-RRA) and characterization of the endogenous inhibitor of GABA binding were investigated.

MATERIALS AND METHODS

Optimum Conditions for GABA-RRA

Receptor preparation. For the receptor preparation, crude synaptic membrane was prepared from whole rat brain using a modification of the method of De Robertis *et al.* (14). Briefly, Sprague-Dawley rats (body weight 200-250 g) were decapitated and the whole brains were immediately removed. The tissue was homogenized in 10 vol of ice-cold 0.32 M sucrose with a Brinkman Polytron PT-10 homogenizer for 20 sec, and the homogenate was centrifuged at 900 g for 10 min. The supernatant was further centrifuged at 11,500 g for 20 min and the pellet (P_2 fraction) was resuspended in 10 vol of Tris-HCl buffer (50 mM, pH 7.6) and kept at -70℃ until assay. Before analysis, the receptor preparation was thawed and homogenized with a glass-homogenizer in 100 vol of Tris-HCl buffer, then centrifuged at 50,000 g for 20 min. The supernatant was discarded and the pellet was resuspended in 100 vol of Tris-HCl buffer containing 0.05 % Triton X-100, and the suspension was shaken in a water bath at 37℃ for 30 min, then centrifuged twice at 50,000 g for 20 min. The pellet was resuspended in 20 vol of Tris-HCl buffer to make a tissue concentration of approximately 0.6 mg protein/ml.

GABA-RRA. For the GABA-RRA, 0.5 ml of the receptor preparation was placed into a glass tube containing 0.3 ml of Tris-HCl buffer, 0.1 ml of specimen and 0.1 ml of [^{3}H]-GABA (Amersham, specific activity 57 Ci/mmol, final concentration 6.4 nM). The samples were incubated in ice for 30 min and the reaction was terminated by filtration through glass fiber filters (Whatman GF/C) under reduced pressure. Each filter was washed twice with 5 ml of cold Tris-HCl buffer and then placed into scintillation fluor for counting. In all experiments, to determine the specific binding, the non-specific component, which was the radioactivity bound to the membrane preparation in the presence of excess GABA (1 mM), was subtracted from the total binding activity. All samples were analyzed in duplicate. Protein concentration was measured by the method of Lowry *et al.* (15).

Regional distribution of GABA receptor in rat brain. The rat brain was dissected into 9 regions (cerebral cortex, corpus striatum, thalamus, hypothalamus, hippocampus, mid brain, pons and medulla oblongata, olfactory bulb and cerebellum) by the method of Glowinski *et al.* (16). Then, the amount of [^{3}H]-GABA binding was measured in each region following the above-described method.

Effects of various drugs on [^{3}H]-GABA binding. The potency of various drugs in displacing specifically bound [^{3}H]-GABA with Triton X-100 treated brain membrane was analyzed.

Characteristics of the Endogenous Inhibitor of GABA Receptor Binding

Effects of the number of centrifugation times on [^{3}H]-GABA binding. Three different kinds of receptor preparation were obtained from stored P_2 fractions. In the first preparation, the P_2 fraction was homogenized in 100 vol of Tris-HCl buffer and centrifuged at 50,000 g for 20 min, and the pellet was resuspended in 20 vol of Tris-HCl buffer. In the second, the P_2 fraction was similarly centrifuged twice, and the pellet was resuspended in 20 vol of Tris-HCl buffer. In the third, the P_2 fraction was centrifuged twice and the pellet was resuspended in

100 vol of Tris-HCl buffer (without Triton X-100) and shaken in a water bath at 37℃ for 30 min, then centrifuged once more. The pellet was resuspended in 20 vol of Tris-HCl buffer. Using each receptor preparation, GABA-RRA was performed and a standard curve and Scatchard plot for each was drawn.

Purification of the endogenous inhibitor. In the standard course of receptor preparation, after centrifugation of the stored P_2 fraction of rat brain, the pellet was resuspended in 100 vol of Tris-HCl buffer containing 0.05 % Triton X-100 and shaken at 37℃ for 30 min, then centrifuged at 50,000 g for 20 min. The supernatant was lyophilized and used as the endogenous inhibitor. The effect of the endogenous inhibitor and GABA on [^{3}H]-GABA binding to receptor preparation was examined.

Interaction between the endogenous inhibitor and diazepam in GABA-RRA. To study the effect of the endogenous inhibitor on [^{3}H]-GABA binding, 7.0 μg protein of the endogenous inhibitor was added to the incubation medium. To examine the interaction between the endogenous inhibitor and diazepam on [^{3}H]-GABA binding, 8×10^{-6} M diazepam and 7.0 μg protein of the endogenous inhibitor were added to the incubation medium. A standard curve and Scatchard plot was drawn in both experiments. A Lineweaver-Burk plot of GABA-RRA was prepared from the data obtained in the absence of the endogenous inhibitor and diazepam, in the presence of the endogenous inhibitor (7.0 μg protein) and in the presence of the endogenous inhibitor (7.0 μg protein) and diazepam (8×10^{-6} M).

Effect of diazepam on [^{3}H]-GABA binding in the presence of GABA or the endogenous inhibitor. A change of [^{3}H]-GABA binding was investigated by increasing the concentration of diazepam up to 10^{-5} M in the presence of GABA (13 nM) or the endogenous inhibitor (7.0 μg protein) causing the same inhibition of binding in the standard curve of GABA-RRA.

Estimation of the molecular weight of the endogenous inhibitor. A Sephadex G-75 column (1.2 × 80 cm) was calibrated using ribonuclease A (M.W. 13,700) and 2-mercaptoethanol (M.W. 78.1). The column was equilibrated with Tris-HCl buffer (50 mM, pH 7.6) and used at 4℃. In 2 ml of Tris-HCl buffer, 1.12 mg protein of the endogenous inhibitor was dissolved and applied to a Sephadex G-75 column. Fractions of 3.0 ml were collected. Blue dextran was added to mark the void volume and 2-mercaptoethanol to mark the total volume. The fractions containing the endogenous inhibitor were determined by adding 0.2 ml of each fraction to the GABA-RRA incubation medium and then measuring the percent inhibition of [^{3}H]-GABA binding.

RESULTS

Optimum Conditions for GABA-RRA

Concentration of receptor preparation. The [^{3}H]-GABA binding increased linearly in the receptor concentration range of 100 to 400 μg protein/tube, and accordingly, a receptor concentration of 300 μg protein/tube was used in this study.

Incubation time. The [^{3}H]-GABA binding reached its maximal level at 30 min of incubation time and remained constant for up to 100 min. In the standard GABA-RRA, the incubation time was set at 30 min.

Difference between GABA-RRA and muscimol-RRA. Fig. 1 shows the standard curve and Scatchard plot of GABA-RRA and muscimol-RRA. Scatchard analysis revealed the presence of two populations of binding sites, the high affinity binding site having an apparent dissociation constant (Kd) of 11.7 nM with a maximal

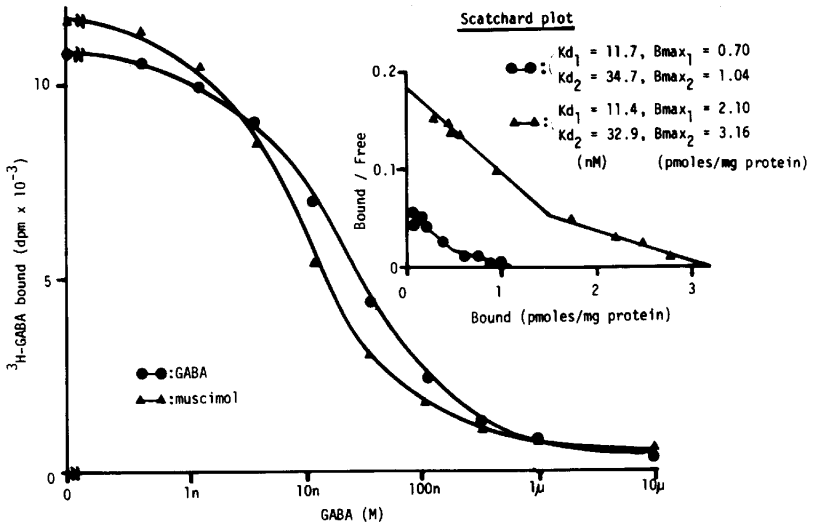

Fig. 1. The standard curve of GABA-RRA (●—●) and muscimol-RRA (▲—▲). The insert shows the same data plotted as a Scatchard plot.

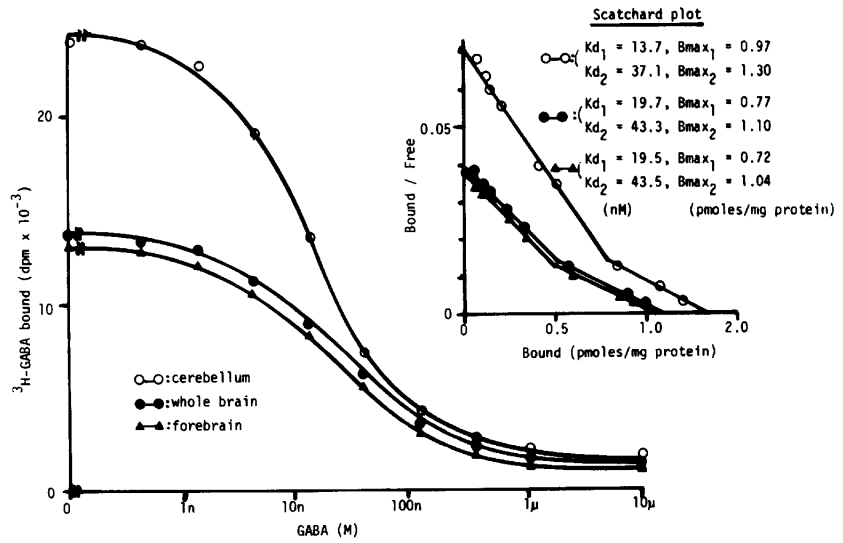

Fig. 2. The standard curve of GABA-RRA using synaptic membrane from rat cerebellum (○—○), whole brain (●—●) and forebrain (▲—▲). The insert shows the same data plotted as a Scatchard plot.

binding capacity (Bmax) of 0.7 pmoles/mg protein, and the low affinity binding site having a Kd of 34.7 nM with a Bmax of 1.04 pmoles/mg protein in GABA-RRA. In muscimol-RRA, high and low affinity binding sites were also present,

the former having a Kd of 11.4 nM with a Bmax of 2.1 pmoles/mg protein and the latter having a Kd of 32.9 nM with a Bmax of 3.16 pmoles/mg protein. In both assays, the Kd of high and low affinity binding sites were similar, but the Bmax of muscimol-RRA was about 3 times larger than that of GABA-RRA, indicating that muscimol might label not only GABA receptors but other unknown receptors as well.

GABA-RRA in rat whole brain, forebrain and cerebellum. As shown in Fig. 2, the specific [^{3}H]-GABA binding in the cerebellum was considerably higher than that in the whole brain or forebrain, which was due to the high Kd and Bmax. In the rat whole brain and forebrain, the specific [^{3}H]-GABA binding were similar.

Regional distribution of GABA receptors in rat brain. The highest level of specific [^{3}H]-GABA binding was found in the cerebellum which had about 1.6 times the binding of the next highest region, the olfactory bulb (Table 1). The levels of GABA binding were similar in the hippocampus and cerebral cortex. The lowest level of binding was detected in the pons and medulla oblongata, which was about 7.2 % that of the cerebellum.

Effects of various drugs on specific [^{3}H]-GABA binding. The potency of various drugs in displacing specifically bound [^{3}H]-GABA was analyzed (Table 2). Imidazole acetic acid, a potent GABA agonist, was effective in displacing [^{3}H]-GABA binding. β-alanine inhibited [^{3}H]-GABA binding very little. Most of the other drugs did not interfere with [^{3}H]-GABA binding.

Characteristics of the Endogenous Inhibitor of GABA Receptor Binding

Effects of the number of washing times on [^{3}H]-GABA binding. As shown in Fig. 3, the Scatchard plot revealed that the receptor preparation with one or two washings of the P_2 fraction had only a low affinity binding site. On the other hand, high and low affinity binding sites were detected (Kd = 63.8 nM, 135 nM) in the receptor preparation when the P_2 fraction was washed three times and shaken once. These data indicated that the endogenous inhibitor of GABA binding could be

TABLE 1. REGIONAL DISTRIBUTION OF GABA RECEPTORS IN THE RAT CENTRAL NERVOUS SYSTEM

Region	Specific binding of [^{3}H]-GABA (fmoles/mg protein)
Cerebellum	264 ± 69
Olfactory bulb	162 ± 48
Hippocampus	133 ± 42
Cerebral cortex	129 ± 10
Hypothalamus	77 ± 10
Corpus striatum	67 ± 23
Midbrain	46 ± 5
Thalamus	33 ± 7
Pons + medulla oblongata	19 ± 4

Data were shown as means ± SEM of 3 experiments.

TABLE 2. EFFECTS OF VARIOUS DRUGS ON SPECIFIC [^{3}H]-GABA BINDING

Drug	IC_{50} (nM)
GABA	14
Baclofen	*
Hopantenate calcium	5,100
L-glutamic acid	*
Imidazole acetic acid	400
Picrotoxin	*
Bicuculline	*
β-alanine	1,500
Anticholinergic drugs	
Profenamine HCl	*
Atropine sulfate	79,000
Levodopa	53,000
Carbidopa	*
Amantadine HCl	*
Bromocriptine	*
Citicoline	*
Sulpiride	*
Trimipramine maleate	34,000
Chlorpromazine HCl	32,000
β-blocker	
Propranolol HCl	*
Oxprenolol HCl	*
Pindolol	*

* : no effect at 100 μM

Each IC_{50} determination used four or six concentrations of drugs in duplicate in competing standard GABA-RRA. Results were converted to percentages of maximal specific binding and plotted on log-probit paper to estimate the concentration of 50 % binding inhibition.

partially removed from rat P_2 fractions by only washing and shaking without treatment with Triton X-100.

Effect of Triton X-100 treatment on [^{3}H]-GABA binding. Three different concentrations of Triton X-100 were used for receptor preparations. High and low affinity binding sites and capacities were similar in these three cases (Fig. 4). The treatment with Triton X-100 significantly increased the binding affinity and capacity.

Purification of the endogenous inhibitor. The effects of GABA and the endogenous inhibitor on [^{3}H]-GABA binding were dose-dependent and the slope of the dilution curves were identical, indicating that the endogenous inhibitor took competitive action with GABA for GABA binding sites (Fig. 5).

Interaction between the endogenous inhibitor and diazepam in GABA-RRA. In the presence of 7.0 μg protein of the endogenous inhibitor, [^{3}H]-GABA binding was

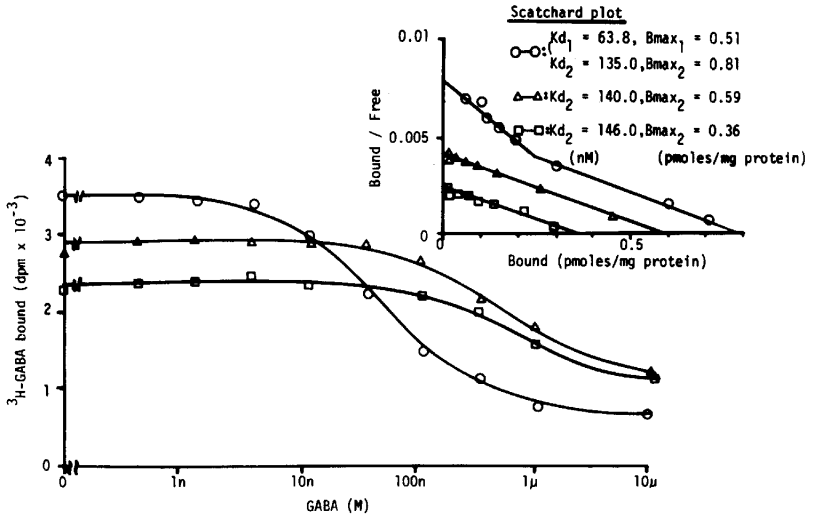

Fig. 3. The standard curve of GABA-RRA, using three different kinds of receptor preparation obtained from rat P_2 fractions by once washing (50,000 g for 20 min) (□—□), twice washing (△—△) and three times washing and once shaking (37 ℃ for 30 min) in the standard Tris-HCl buffer without Triton X-100 (○—○).

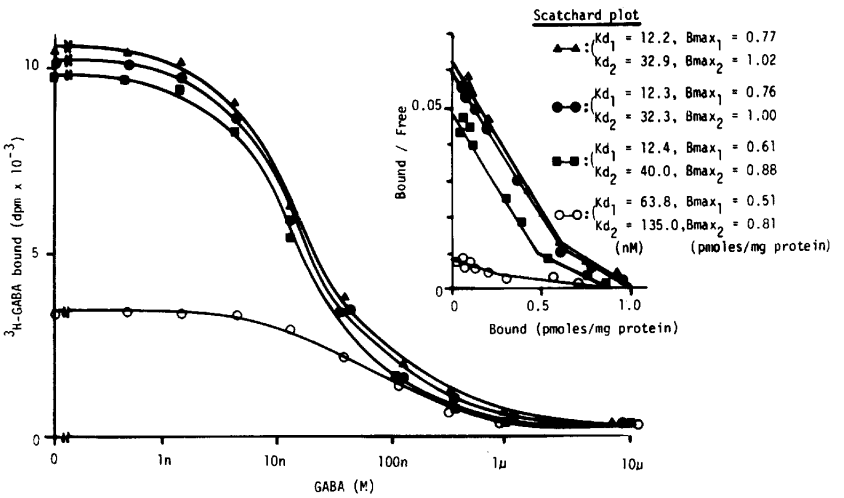

Fig. 4. The standard curve of GABA-RRA, using receptor preparations treated with 0.1 % (▲—▲), 0.05 % (●—●), 0.025 % (■—■) Triton X-100 and not treated with Triton X-100 (○—○).

inhibited to almost 50 % of the control (Fig. 6). The Scatchard analysis revealed the existence of only a low affinity binding site (Kd = 47.9 nM). In the presence of 7.0 μg protein of the endogenous inhibitor and 8×10^{-6} M diazepam, however, the [^{3}H]-GABA binding was decreased by an amount equal to only 30 % of the

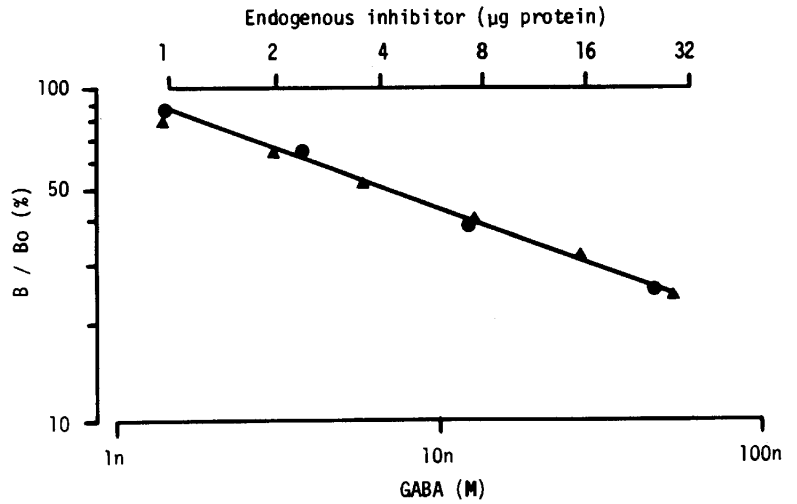

Fig. 5. Effect of GABA (●—●) and an endogenous inhibitor (▲—▲) on [^{3}H]-GABA binding. Percent inhibition of specific [^{3}H]-GABA binding was determined and plotted on log-probit paper.

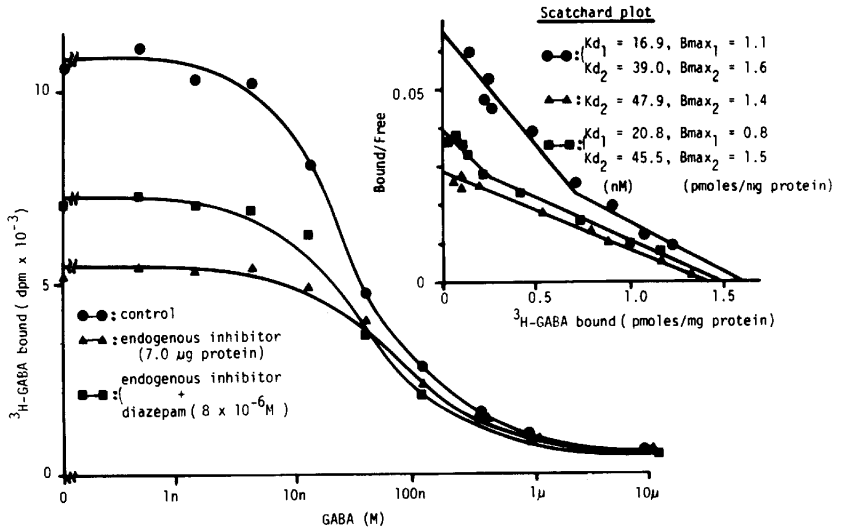

Fig. 6. The standard curve of GABA-RRA in the absence of an endogenous inhibitor and diazepam (●—●), in the presence of 7.0 μg protein of an endogenous inhibitor (▲—▲) and in the presence of 7.0 μg protein of an endogenous inhibitor and 8×10^{-6} M diazepam (■—■).

control bound. The 20 % regain of GABA binding was due to the reappearance of a high affinity binding site (Kd = 20.8 nM). These data suggested that the inhibition due to the endogenous inhibitor was blocked by diazepam. A Lineweaver-Burk plot clarified the interaction between the endogenous inhibitor and diazepam, namely that diazepam gave allosteric effects to GABA binding sites

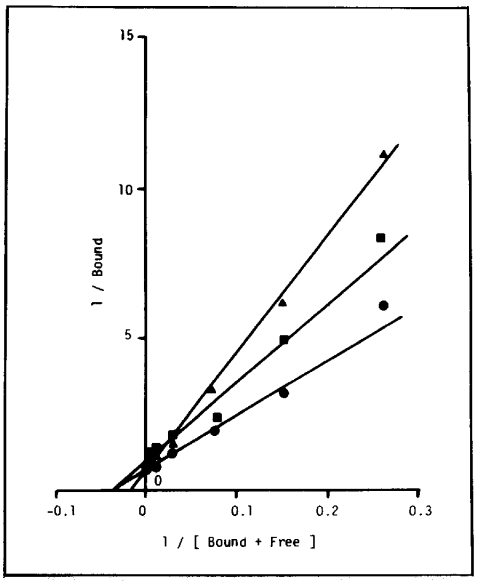

Fig. 7. Lineweaver-Burk plot of the same data in Fig. 6.

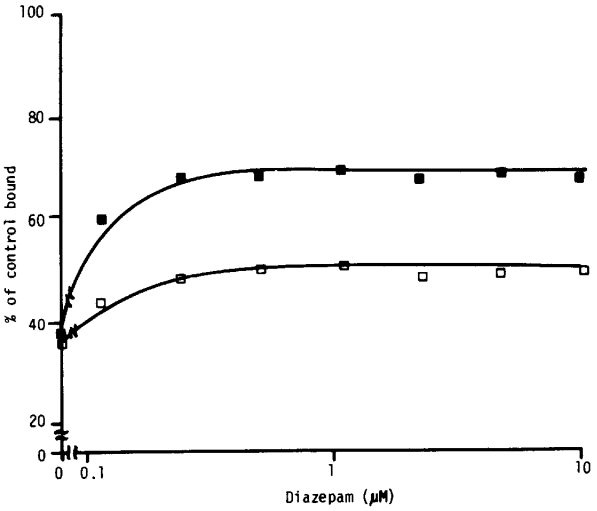

Fig. 8. Effect of diazepam on specific [^{3}H]-GABA binding in the presence of GABA (13 nM) (□–□) or an equal potency of an endogenous inhibitor (7 μg protein) (■–■).

which then blocked the competitive action of the endogenous inhibitor (Fig. 7).

Effect of diazepam on [^3H]-GABA binding in the presence of GABA or the endogenous inhibitor. In the present GABA-RRA, diazepam up to 10^{-5} M had no effect on [^{3}H]-GABA binding. When increasing the concentration of diazepam in the

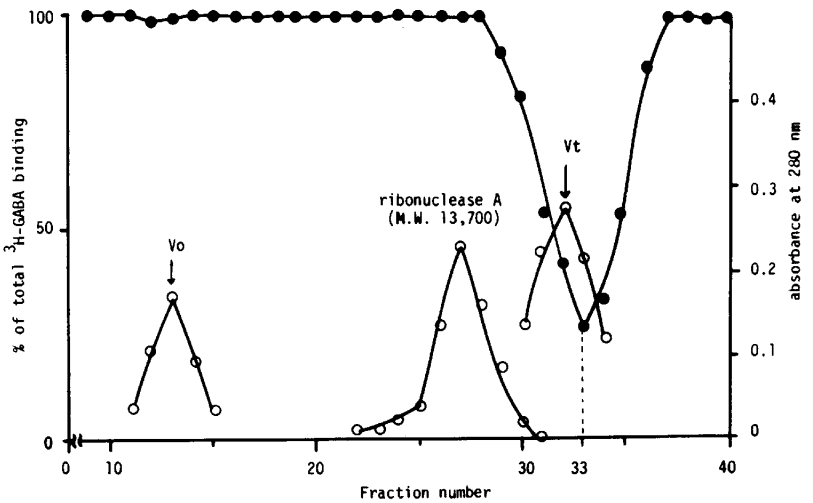

Fig. 9. Sephadex G-75 elution profile of the endogenous inhibitor. The blue dextran was added to mark the void volume (Vo) and 2-mercaptoethanol to mark the total volume (Vt). The elution volume was determined by absorbance at 280 nm (○). The fraction containing the endogenous inhibitor was determined by measuring the percent inhibition of [³H]-GABA binding (●).

presence of 7.0 μg protein of the endogenous inhibitor or 13 nM GABA which inhibited binding to the same degree in the standard curve, [³H]-GABA binding was increased in proportion to the concentration of diazepam up to 3.2×10^{-7} M and reached a plateau over this level (Fig. 8). In the presence of diazepam, [³H]-GABA binding with the endogenous inhibitor was higher than that with GABA, whereas there was no difference in the absence of diazepam.

Estimation of the molecular weight of the endogenous inhibitor. Fig. 9 shows the distribution of the endogenous inhibitor after gel filtration of Sephadex G-75. The endogenous inhibitor was eluted at fraction number 33 which had the most potent inhibitory activity on [³H]-GABA binding, and the molecular weight of the endogenous inhibitor was estimated to be less than 3,000 daltons.

DISCUSSION

Recently, the investigation of neurotransmitters by radioreceptor assay has made remarkable progress. Muscimol is a naturally occurring GABA analogue found in Amantia muscaria. It was reported that muscimol had a higher affinity for the GABA receptor than GABA and that it could more selectively label the postsynaptic receptor than the uptake site (17). Therefore, many investigations of the GABA receptor have been performed using [³H]-muscimol instead of [³H]-GABA (9, 10, 18, 19). As shown in Fig. 1, the maximal binding capacity (Bmax) of muscimol-RRA was 3 times larger than that of GABA-RRA. This finding suggested that muscimol might label not only GABA receptors but also other un-

known receptors. Therefore, [^{3}H]-GABA should probably be used as a ligand to detect the GABA receptor. De Feudis *et al.* (20) recently reported that there might be an extrasynaptic binding site for muscimol which could not be occupied by GABA. For these reasons, [^{3}H]-GABA was used for studying GABA receptors in the present study.

The highest concentrations of GABA receptors were observed in the rat cerebellum, olfactory bulb, hippocampus and cerebral cortex (Fig. 2, Table 1), indicating that GABA played an important role in these areas of the CNS.

Most of the tested drugs had little or no effect on [^{3}H]-GABA binding except imidazole acetic acid, β-alanine and hopantenate calcium. A functional link between the GABA, dopamine and cholinergic systems in the basal ganglia has been suggested, and it was reported that GABA was inhibitory on the nigrostriatal dopamine system and that GABA neurons which influenced dopaminergic activity were regulated, at least in part, by the cholinergic system (21, 22). However, the results of this study suggested that anti-parkinsonian drugs had little or no relation with GABA receptors (Table 2). Enna (23) reported that GABA mimetics appeared to be contraindicative in Parkinson's disease because of their inhibitory effect on the nigrostriatal dopaminergic system. Lloid *et al.* (24) demonstrated that a significant decrease in [^{3}H]-GABA binding was observed in the substantia nigra of patients with Parkinson's disease, and they suggested that there might be [^{3}H]-GABA binding sites on the cell bodies (or dendrites) of the nigral dopamine neurons. Therefore, GABA mimetics may not be so contraindicative in Parkinson's disease as a result of the decrease in GABA receptors in the substantia nigra.

Recently, Toffano *et al.* (13) reported the existence of an endogenous inhibitor in rat synaptic membranes, which regulated the affinity of GABA receptor binding. They also revealed that it was a small thermostable protein and its molecular weight was about 15,000 daltons. They removed the endogenous inhibitor from synaptic membranes of rat brain by freezing and thawing and by repeated washing with Tris-citrate buffer containing 0.01 % Triton X-100. Since then, there have been many studies of the endogenous inhibitor (11, 25-27). As shown in Fig. 3, high and low affinity binding sites appeared in well-washed receptor preparations without Triton X-100 treatment. The high and low binding affinities and binding capacities, however, dramatically increased with the Triton X-100 treatment (Fig. 4). Greenlee *et al.* reported that pretreatment of the membranes with 0.05 % Triton X-100 resulted in virtually no change in the binding characteristics (11). The difference of the results in these two studies might be due to the difference of the removal ratio of the endogenous inhibitor from synaptic membranes with or without Triton X-100 treatment. GABA enhanced the affinity of benzodiazepine receptors for [^{3}H]-flunitrazepam in rat brain and it was supposed that the GABA effect on benzodiazepine receptors was due to the allosteric effect of GABA or the decrease of an endogenous benzodiazepine-like neurotransmitter which might act at the benzodiazepine binding site (28, 29). On the other hand, ben-

zodiazepines protected [^{3}H]-muscimol binding from inactivation by heat, and in this case GABA receptors had benzodiazepine recognition sites (30). Matsumoto *et al.* (31) demonstrated that diazepam and flunitrazepam (3 μM) increased significantly the specific binding of 40 nM but not 2 nM [^{3}H]-muscimol and that this stimulation was attributed to an increase of the low affinity component of GABA receptors. They concluded that only low affinity GABA receptor sites were linked to benzodiazepine receptors. In the present standard GABA-RRA, diazepam had no effect on [^{3}H]-GABA binding up to 10^{-5} M. Diazepam (8×10^{-6} M), however, partially blocked the inhibitory action of the endogenous inhibitor (7.0 μg protein) which was attributed to the allosteric effect of diazepam on GABA receptors as shown in Fig. 6 and Fig. 7. Recently, a GABA/benzodiazepine/Cl^- ionophore complex was proposed (32, 33). On the basis of the hypothesis, the effect of diazepam on GABA receptors could be explained as follows. Diazepam first binds to benzodiazepine receptors and then an allosteric change in GABA receptors arises which opposes the inhibitory effect of the endogenous inhibitor. Various experiments have demonstrated the molecular weight of the endogenous inhibitor of GABA receptor binding to be about 15,000 (13), to less than 500 daltons (26). The molecular weight of the endogenous inhibitor was found to be less than 3,000 daltons by gel filtration (Fig. 9) in this study. Some groups (25, 27) reported that the endogenous substance might be GABA itself. In this study, therefore, an experiment was attempted to resolve the problem of whether or not the endogenous inhibitor was GABA itself. The data (Fig. 8) indicated clearly that the endogenous inhibitor was different from GABA. If the endogenous inhibitor was GABA itself, [^{3}H]-GABA binding to receptor preparation with GABA should have been the same as that with an equal inhibitory potency of the endogenous inhibitor. In the present study, it was clearly demonstrated that the endogenous inhibitor was not GABA itself. The extraction and refining of the endogenous inhibitor is under active investigation.

Ackonwledgment. The author wishes to thank Prof. Zensuke Ota for his kind guidance and critical review of this manuscript and Dr. Norio Ogawa for his helpful advice and encouragement during this study.

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